BRCAI AND hMLHI GENE PRIMER SEQUENCES AND METHOD FOR TESTING

The present application is based upon provisional application 60/084408, filed May 6, 1998, and is directed to methods of and primer sequences for sequence variation and/or mutation detection of BRCA and hMLH1 genes , such as by two-dimensional denaturing gradient electrophoresis techniques (TDGS).

Background

Such techniques are described in Method Of And Apparatus For Diagnostic DNA Testing, Jan Vijg and Daizong Li, PCT/IB96/00543, filed 3 June 1996, International Publication Number WO96/39535, 12 December 1996, and in "Two-Dimensional DNA Typing", Molecular Bio Technolgoy, Vol. 4, 1995, pp 275-295.

The tests leading to the establishment of the primer sequences for the BRCAI and hMLH1 of the present invention were conducted with the TDGS design prepared with the computer programming and equipment described in PCT/IB97/00976, published on or about February 14, 1998.

Objects of Invention and Summary

The objects of the invention are to provide novel theoretically and empirically (experimentally) derived TDGS patterns for hMLH1 and BRCA1 genes which may be used by testers to test for gene sequence variation and/or mutations.

Drawings

Figs. 1A and 1B show the computer-aided design TDGS patterns obtained for the hMLH1 and BRCA1 (theoretical-left hand side; empirical or experimental--right hand side).

In the theoretical vs. empirical patterns of the MLHI and BRCA1 genes, for all four genes, one or more exons were designed in overlapping fragments, in which case the fragment name is exon.1, exon..2, etc. Exons 8 and 15 of hMLH1 contain polymorphisms, which can be distinguished from disease-causing heterozygous mutations on the basis of a unique four-spot pattern (18).

Description Of The Invention In Preferred Forms

The MLH1 DNA mismatch repair gene. The design for *MLH1* took 30 minutes (excluding exon indication). Fig. 1A shows the theoretical and the empirical TDGS pattern for the *MLH1* gene. Because exons 11 and 12 had to be subdivided into overlapping fragments, two multiplex groups are currently being used, with the long PCR carried out as a four-plex PCR. Like many other genes, exon 1 of *MLH1* is GC-rich and, hence, was found to melt at a much higher % UF compared to most of the other fragments. Thus far, a total of 41 coded samples with previously identified mutations have been analyzed in a blinded fashion with 100% concordance (30).

The breast and ovarian cancer susceptibility gene BRCA1. The tumor suppressor gene *BRCA1* contains 24 exons, of which exon 11 contains approximately 60 % of the coding region. Fig. 1B shows the theoretical and empirical 2-D pattern for *BRCA1*. Of all 2-D designs discussed, this was the most difficult (total design time was 2 h), the main reason being the need to make overlapping fragments for the 3.4 kb exon 11. Pre-amplification was accomplished by one 7-plex long PCR. Using the long PCR amplicons as template, all 24 exons were amplified in a total of 37 fragments distributed over 5 multiplex groups. The overlap and sometimes short distances from fragment to fragment necessitated the use of so many multiplex short PCR groups. The non-coding exons 1a, 1b and the non-coding part of exon 24 were excluded. Evaluation of this test design using a panel of coded samples with previously identified mutations is currently ongoing. Thus far, mutations and polymorphisms have been detected in exons 2, 8, 11, 16, 20 and 23.

PCR Amplification

Primers were obtained from Genosys Biotechnologies, Inc. (The Woodlands, TX). For complete lists of all sequences, except *BRCA1*, see references 18, 29 and 30. Primer sequences for *BRCA1* will be published elsewhere but will be made available upon request. PCR amplification of gene sequences was carried out using the two-step protocol first described by Li and Vijg (22). Primers for long-distance PCR were designed based on published sequences (24-27) using Primer Designer 3, to amplify the entire gene-coding region for each of the 4 genes as a 1-plex (*TP53*), a 6-plex PCR (*RB1*), a 4-plex PCR (*MLH1*) or a 7-plex PCR (*BRCA1*). The LA PCR kit (Takara) was used for long PCR in a PTC-100 thermocycler (MJ Research). Multiplex short PCR was carried out using the long PCR products as template. Between 0.1 and 1.125 µM of each primer was used in a 50 µl reaction with 1 µl of long PCR product in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 250 µM of each dNTP and 5 % formamide. Two and a half units of Taq DNA polymerase (Life Technologies) were added after an initial denaturation at 94 °C for 60 s. Cycling conditions for multiplex short PCR and concentrations of $MgCl_2$ varied among different genes and amplifications were carried out in a PTC-100 thermocycler (MJ Research).

Two-dimensional DNA electrophoresis

For *RB1*, 5 µl of multiplex short PCR was used per electrophoresis run. For *TP53*, *MLH1* and *BRCA1*, 5 µl of each of the different multiplex groups were combined. One tenth of a

volume of loading buffer (0.25 % xylene cyanol, 0.25 % bromophenol blue, 15 % ficoll and 100 mM Na_2EDTA) was added and the mixtures were loaded onto a 6.5 % (*TP53*) or 10 % (*RB1*, *MLH1* and *BRCA1*) PAA non-denaturing size gel (acrylamide: bisacrylamide = 37.5:1) in 0.5 x TAE buffer. The samples were electrophoresed for 5.3 h at 150 V (*RB1*), 5 h at 120 V (*TP53*) or 7.5 h at 140 V (*MLH1* and *BRCA1*) at 50 °C. After staining the gel with a mixture of equal amounts of SYBR-green I and II (Molecular Probes, Eugene, Oregon) for 20 min, the region containing all fragments of interest (usually between 100 and 600 bp) was cut out and loaded onto a denaturing gradient gel (DGGE). Gradients used were 0 to 50 % UF for *RB1*, 20 to 70 % UF for *TP53*, 25 to 70 % UF for MLH1 and 20 to 65% UF for BRCA1. The second orthogonal dimension was run for 12 h at 100V (RBI), 14 h at 120 V (TP53) or 16 h at 100 V (MLH1 and BRCA1). Spot patterns were visualized by SYBR-green staining using a FluorImager (Molecular Dynamics, Sunnyvale, California).

The primer sequences for long and short PCR for the BRCA1 are as follows:

A. Primer Pairs for Long Distance PCR

Exons 1-4

MLH1-4F GCG.GCT.AAG.CTA.CAG.CTG.AAG.GAA.GAA.CGT.GA

MLH1-4R GGC.GAG.ACA.GGA.TTA.CTC.TGA.GAC.CTA.GGC.CC

product size= 10.8kb

Exons 5-10

MLH5-10F

GCG.CCC.CTT.GGG.ATT.AGT.ATC.TAT.CTC.TCT.ACT.GG

MLH5-10R GCG.CTC.ATC.TCT.TTC.AAA.GAG.GAG.AGC.CTG

product size=10.5kb

Exons 11-13

MLH11-13F CGG.CTT.TTT.CTC.CCC.CTC.CCA.CTA.TCT.AAG.G

MLH11-13R GGG.TTA.GTA.AAG.GAA.GAG.GAG.CTT.GCC.C

product size=8.7kb

Exons 14-19

MLH14-19F GGT.GCT.TTG.GTC.AAT.GAA.GTG.GGG.TTG.GTA.G

MLH14-19R

GCG.CGC.GTA.TGT.TGG.TAC.ACT.TTG.TAT.ATC.ACA.C

product size=10.5kb

Underlined nucleotides represent nucleotides added to modify melting temperatures of the primers

B. Primer Pairs for Short PCR

Exon	Clamp[1]	Product Size	Tm[2]	Primer Sequence

12.1	40	184	44.53	TTT.TTT.TTT.TTT.TAA.TAC.A
				AAT.CTG.TAC.GAA.CCA.TCT
12.2	8	366	53.23	TGG.AAG.TAG.TGA.TAA.GGT
	40			TGT.ACT.TTT.CCC.AAA.AGG
13	40	272	49.06	ATC.TGC.ACT.TCC.TTT.TCT
				AAA.ACC.TTG.GCA.GTT.GAG
14	45	235	48.94	TAC.TTA.CCT.GTT.TTT.TGG
	5			GTA.GTA.GCT.CTG.CTT.GTT
15	40	179	29.97	CAG.CTT.TTC.CTT.AAA.GTC
				CAG.TTG.AAA.TGT.CAG.AAG
16		261	47.56	CTT.GCT.CCT.TCA.TGT.TCT.TG
	40			AGA.AGT.ATA.AGA.ATG.GCT.GTC
17	40	199	47.01	ATT.ATT.TCT.TGT.TCC.CTT
				AAT.GCT.TAG.TAT.CTG.CCT
18	45	215	46.67	CCT.ATT.TTG.AGG.TAT.TGA.AT
				GCC.AGT.GTG.CAT.CAC.CA
19.1		282	43.43	TGT.TGG.GAT.GCA.AAC.AGG
	40			ATC.CCA.CAG.TGC.ATA.AAT

[1]GC clamps:

50 clamp:

CGC.CCG.CCG.CCG.CCC.GCC.GCG.CCC.CGC.GCC.CGT.CCC.GCC.GC

C.CCC.GCC.CG

45 GC clamp:

claims 27-32

CGC.CCG.CCG.CGC.CCC.GCG.CCC.GTC.CCG.CCG.CCC.CCG.CCC.GG
C.CCG

40 clamp:

CGC.CCG.CCG.CGC.CCC.GCG.CCC.GGC.CCG.CCG.CCC.CCG.CCC.G

8 clamp:

CGT.CCC.GC

5 clamp:

GCG.CG

2 clamp:

CG

[2]Tm is given in %UF

The primer sequences for long and short PCR for the BRCA1 are as follows:

Primers for long-PCR BRCA1
(7-PLEX)

BR1/1-3F : TgT ACC TTg ATT TCg TAT TCT gAg Agg CTg CTg CTT Ag
BR1/1-3R : gAg AAA gAA TgA AAT ggA gTT ggA TTT TCg TTC TCA C

Size: ***9.9 kb***

BR1/5-9F : TAg CCA TgA AAA gAT AAT CTC ACA ACT gCC CTT AAg AgC
BR1/5-9R : ACC AgC CTA CTT gAg ggA ggA Agg Tgg gAA gA

Size: ***9.7 kb***

BR1/10-11F : gAg AgC AgC TTT CAC TAA CTA AAT AAg ATT ggT CAg CTT TCT gT
BR1/10-11R : TCA AgT TTA AgA AgC AgT TCC TTT AAC TAT ACT Tgg AAA TTT gT

Size: ***4.8 kb***

BR1/12-13F : gCT Agg ACg TCA TCT TTg gCT TgA ATg AgC TTT A
BR1/12-13R : gCg ATA ATT ACC CAT gTg CTg AgC AAg gAT CA

Size: ***9.0 kb***

BR1/14-17F : TCT TCA ATg Tgg Agg CAg TAg ggA Tgg AgA AA
BR1/14-17R : ggg TCT CCA ggT TTT gCC TCA CTT gTT CTT TC

Size: ***10.7 kb***

BR1/18-20F : TCT TAA CTT CAT ATC AgC CTC CCC TAg ACT TCC AAA TAT CC
BR1/18-20R : CAT CTC TgC AAA ggg gAg Tgg AAT ACA gAg Tg

Size: ***7.2 kb***

BR1/21-24F : CAC TCT TCC ATC CCA ACC ACA TAA ATA AgT ATT gTC TC
BR1/21-24R : gCA TAg CCA gAA gTC CTT TTC Agg CTg ATg TAC AT

Size: ***11.4 kb***

10 Exon 11

BC1EX11

Exon	Frag	Primers 5' -> 3'		size	Tm(°C)
11.1	[GC 3]	ACCTTGTTATTTTTGTATATTT	22	347	40.99
	[GC 13]	TTGCTAAGCCAGGCTGTT	18		
11.2	[GC 3]	ATACTCATGCCAGCTCATTA	20	461	40.74
	[GC 12]	AACGTCCAATACATCAGCTA	20		
11.3		CATGCTCAGAGAATCCTAGA	20	438	35.04
	[GC 3]	CTGTGGCTCAGTAACAAATG	20		
11.4	[GC 12]	TCACTCCAAATCAGTAGAGA	20	476	34.85
	[GC 3]	TACTGCTGCTTATAGGTTCA	20		
11.5	[GC 3]	GAAAGCAGATTTGGCAGTTC	20	468	33.66
	[GC 11]	CTGACTGGCATTTGGTTGTA	20		
11.6	[GC 3]	GAATAGGCTGAGGAGGAAGT	20	410	40.51
	[GC 13]	CTCTTGGAAGGCTAGGATTG	20		
11.7	[GC 3]	ACAGCGATACTTTCCCAGAG	20	345	36.45
		TGCCTTCCCTAGAGTGCTAA	20		
11.8		TTGCAAACTGAAAGATCTGT	20	365	38.37
	[GC 3]	GCTTTGAAACCTTGAATGTA	20		
11.9	[GC 13]	GTCGGGAAACAAGCATAGAA	20	422	40.40
	[GC 4]	TTGCCTCTGAACTGAGATGA	20		
11.10	[GC 12]	TAATATCACTGCAGGCTTTC	20	292	35.93
	[GC 1]	TTCCTCAAAGTTTTCCTCTA	20		
11.11	[GC 1]	TCCCATCAAGTCATTTGTTA	20	390	33.06
		TTCCAGGAAGACTTTGTTTA	20		
11.12	[GC 12]	TAATGAAGTGGGCTCCAGTA	20	309	33.22
	[GC 1]	CTTCCCATAGGCTGTTCTAA	20		
11.13	[GC 1]	GCAAGAATATGAAGAAGTAG	20	305	37.43
		CAAATGTGTATGGGTGAAAG	20		
11.14	[GC 1]	AGACACCTGATGACCTGTTA	20	378	43.03
	[GC 12]	TCTCCTCTGTGTTCTTAGAC	20		
11.15		CCTTTCACCCATACACATTT	20	460	39.33
	[GC 8]	GACTGATGCCTCATTTGTTT	20		
11.16	[GC 3]	CTCAGGAACATCACCTTAGT	20	356	44.00
	[GC 16]	ATAAATAGACTGGGCCACAC	20		

All exons excluding exon 11

BRCAONE

Exon	Frag	Primers 5' -> 3'		size	Tm(%UF)
2	1	[GC 1]TATATATGTTTTTCTAATGTGT	22		
		[GC 12]TCCCAAATTAATACACTCTT	20	203	34.64
3	1	[GC 12]GAGCCTCATTTATTTTCT	18		
		[GC 4]ATTTTTCGTTCTCACTTA	18	269	37.22
5	1	[GC 4]TATTTGCCTTTTGAGTAT	18		
		[GC 12]TCTGATGAATGGTTTTAT	18	305	26.69
6	1	[GC 8]ACTTGCTGAGTGTGTTTC	18		
		GCACTTGAGTTGCATTCT	18	213	35.52
7	1	[GC 3]TACATTTTTCTCTAACTGC	19		
		GAAGAAAACAAATGGTTTT	19	250	32.67
8	1	GGAGGAAAAGCACAGAAC	18		
		[GC 3]CCAGCAATTATTATTAAATACTT	23	248	40.51
9	1	[GC 3]CAGTAGATGCTCAGTAAA	18		
		AATACCAGCTTCATAGAC	18	242	24.26
10	1	[GC 4]CTGCATACATGTAACTAG	18		
		CTACCCACTCTCTTTTCA	18	229	38.30
12	1	[GC 4]AGTTGCAGCGTTTATAGT	18		
		[GC 13]CAGCAAACCTAAGAATGT	18	289	48.54
13	1	[GC 4]GCTTCTCAAAGTATTTCA	18		
		AGTGTTTGGCCAACAATA	18	293	45.18
14	1	[GC 4]CCAATTTGTGTATCATAG	18		
		[GC 13]AGTGTATAAATGCCTGTA	18	417	30.78
15	1	[GC 1]TGGTTTTCTCCTTCCATTTA	20		
		[GC 16]TGTTCCAATACAGCAGATGA	20	303	46.07
16	1	[GC 13]CGTTGTGTAAATTAAACTTC	20		
		[GC 1]AGTCATTAGGGAGATACATA	20	427	47.49
17	1	[GC 4]TGTGCTAGAGGTAACTCA	18		
		[GC 11]CTCATGTGGTTTTATGCA	18	242	32.51
18	1	[GC 12]TTTCAACTTCTAATCCTTT	19		
		[GC 4]GGAGAAATAGTATTATACT	19	194	36.32
19	1	GTTCTTCTGCTGTATGTA	18		
		[GC 4]CTGAATGAATATCTCTGG	18	178	32.32
20	1	[GC 4]CTCTTTCTCTTATCCTGAT	19		
		TGGTGGGGTGAGATTTTT	18	219	46.40
21	1	[GC 8]ATTCCCCTGTCCCTCTCT	18	172	49.95

CTGGAACTCTGGGGTTCT 18

2	1	[GC 4]TGATTTTACATCTAAATGTC 20			
		[GC 13]AGGAGAGAATATTGTGTC 18		209	47.71
3	1	[GC 12]TAGTCCTACTTTGACACT 18			
		[GC 4]AAATATTTAAAATGTGCCAA 20		275	49.47
4	1	[GC 13]AATCTCTGCTTGTGTTCTCT 20			
		[GC 18]ATTTAGTAGCCAGGACAGTA 20		325	59.79